Contraction of the type I IFN locus and unusual constitutive expression of *IFN-α* in bats

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Bats harbor many emerging and reemerging viruses, several of which are highly pathogenic in other mammals but cause no clinical signs of disease in bats. To determine the role of interferons (IFNs) in the ability of bats to coexist with viruses, we sequenced the type I IFN locus of the Australian black flying fox, *Pteropus alecto*, providing what is, to our knowledge, the first gene map of the IFN region of any bat species. Our results reveal a highly contracted type I IFN family consisting of only 10 IFNs, including three functional *IFN-α* loci. Furthermore, the three *IFN-α* genes are constitutively expressed in unstimulated bat tissues and cells and their expression is unaffected by viral infection. Constitutively expressed *IFN-α* results in the induction of a subset of IFN-stimulated genes associated with antiviral activity and resistance to DNA damage, providing evidence for a unique IFN system that may be linked to the ability of bats to coexist with viruses.

interferon | innate immunity | bat immunology

Bats harbor a number of emerging and reemerging viruses, many of which are highly pathogenic in humans and other species, including henipaviruses (Hendra and Nipah), coronaviruses (SARS-CoV), rhabdoviruses (rabies and lyssaviruses), and filoviruses (Ebola and Marburg), but cause no clinical signs of disease in bats (1). In addition, bats are capable of clearing experimental infections in vivo with henipaviruses and lyssaviruses at doses of infection that are lethal in other mammals (2, 3). The mechanisms responsible for the ability of bats to coexist with viruses remain poorly understood (4).

The interferon (IFN) system provides the first line of defense against viral infection in vertebrates. There are three types of IFNs in mammals, designated types I, II, and III, which differ in their amino acid sequences and the receptor complex they signal through. Type I and type III IFNs are induced directly in response to viral infection and are key cytokines capable of inducing an "antiviral state" in infected and neighboring cells. Type I IFNs include IFN-α, IFN-β, IFN-ω, IFN-ε, IFN-ζ, IFN-κ, IFN-τ, and IFN-δ, that signal through the IFN-α receptor (IFN-αR) that consists of IFN-αR1 and IFN-αR2 chains (5).

All type I IFN genes (with the exception of *IFN-κ*) are located within the boundaries of *IFN-β* and *IFN-ε*, which spans ∼400 kb in humans and 360 kb in mice (6–9). Among type I IFNs, IFN-α and IFN-β proteins account for the majority of the antiviral response generated following viral infection (10). IFN-α and IFN-β expression are normally undetectable in the absence of infection but are rapidly induced following viral infection or treatment with synthetic ligands, including dsRNA (11). A low level of constitutively expressed *IFN-α* mRNA has been detected in humans and germ-free mice (12–14). In humans, *IFN-α1* and *IFN-α2* transcripts are present in normal spleen, liver, and kidney (13). However, as the spontaneous IFN-α and IFN-β proteins are expressed at very low levels, even the most sensitive assays fail to detect them (15). In addition to direct antiviral activity (although at a very low level), constitutively expressed IFN-α is believed to play a role in priming the IFN response, rendering cells "ready to go" by stimulating amplified IFN-α/β production in response to viral infection and enhanced responses to other cytokines (16, 17). In the promoter regions of human *IFN-α* genes, three modules that are responsible for binding to IFN regulatory factors (IRFs) 3 and 7 determine the induction profile of different IFN-αs. For constitutively expressed human *IFN-α1*, it is believed that binding of IRF3 to the unique module II (also called module C) in the promoter region leads to weak endogenous expression. The promoter regions of all other human *IFN-α* genes (except *IFN-α13*) use modules I and III for binding to IRF3 or IRF7, respectively (18, 19).

IFN-α and IFN-β proteins bind to the IFN-αR and trigger the phosphorylation of STAT1 and STAT2, which then forms a ternary complex with IRF9 to form the tripartite transcription factor IFN-stimulated gene (ISG) factor 3 (ISGF3) and drives the expression of ISGs (5). However, continuous exposure of cells to a low level of IFN-β, which often occurs in cancers, leads to steady-state increased expression of an unphosphorylated form of ISGF3 (U-ISGF3), which in turn leads to the expression of a subset of ISGs also induced by ISGF3. This response can extend resistance to virus infection and render cells resistant to DNA damage (20).

Significance

Here we provide what is, to our knowledge, the first gene map of the type I IFN region of any bat species with the sequence of the type I IFN locus of the Australian black flying fox, *Pteropus alecto*. The bat IFN locus contains fewer IFN genes compared with any other mammal sequenced to date, including only three *IFN-α* genes. We also demonstrate that bat *IFN-α* genes are constitutively expressed in unstimulated bat tissues and cells and that their expression is unaffected by viral infection. This unusual pattern of *IFN-α* expression has not been described in any other species to our knowledge and has important implications for the role of innate immunity in the ability of bats to coexist with viruses in the absence of disease.

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Data deposition: Nucleotide sequence data have been deposited in the GenBank database [accession nos. KT384435–KT384439 (bat BAC clones 19–21) and KT384440 (cloned 3-kb bat IFN region)]. RNA sequence data have been deposited in the Sequence Read Archive [accession nos. SRP067312 (uninfected HEK293T cells) and SRP067371 (PaKiT03 cells)].

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The U-ISGF3–associated ISGs are driven by distinct IFN-stimulated response elements and include Mx1, ISG15, and STAT1 (20).

Few studies have been performed to understand the mechanisms responsible for the ability of bats to coexist with viruses. The sequencing of two bat genomes (*Pteropus alecto* and *Myotis davidii*) has revealed several genes involved in the DNA repair and innate immunity pathways that have undergone positive selection in bats compared with other mammals, providing evidence that the evolution of flight could have had inadvertent consequences for the innate immune system of bats (21). Studies have also inferred the existence of seven *IFN-α* genes in *Pteropus vampyrus* (22), eight *IFN-α* subtypes (or alleles) in *Dobsonia viridis*, and one *IFN-ω* and *IFN-κ* in *Eptesicus serotinus* (23, 24). However, as only low-coverage bat genomes have been used to identify IFNs for these studies, the exact genome structure of type I IFN family members is yet to be confirmed. Current knowledge on bat type I IFN responses is also very preliminary, with descriptions of type I and III IFN induction following polyinosinic:polycytidylic acid (polyI:C) stimulation of bat cells (25). Evidence for unique expression patterns of IFN-related genes have also been described in *P. alecto*, including the constitutive expression of *IRF7* and a wider distribution of the type III IFN receptor consistent with the constitutive activation of some aspects of the innate immune system (26, 27). In this study, we report what is, to our knowledge, the first systematic characterization of the bat type I IFN locus and comparison with other species. We also describe the unique constitutive expression of *IFN-α* and ISGs in unstimulated bat tissues and cells, a finding that may have implications for the ability of bats to coexist with viruses in the absence of disease.

Results

Sequencing and Annotation of *P. alecto* Type I IFN Genomic Locus. Two scaffolds (scaffold95 and scaffold222) corresponding to the partial type I IFN locus were identified in the *P. alecto* whole-genome sequence (21). Scaffolds 95 and 222 span 25.14 Mb and 4.344 Mb, respectively, and each contains type I IFN genes. However, these scaffolds did not overlap and therefore did not cover the entire type I IFN locus. To obtain the complete sequence of the type I IFN locus, a *P. alecto* BAC library was used to identify the remaining type I IFN region. The BAC library was screened with overgo probes corresponding to *IFN-β*, *IFN-ε* and kelch-like 9 (*KLHL9*), yielding a total of seven BACs corresponding to the IFN region. BAC end sequences were determined for the positive BAC clones using Sanger sequencing to determine whether any of the BAC clones overlapped with each other or with the genomic scaffolds. A total of five BAC clones were chosen for further long-read pyrosequencing and analysis. The five positive BAC clones were assembled into a single scaffold 433 kb in length with a gap of 21 kb, which was filled by cloning (3 kb) and using data from the closely related bat, *P. vampyrus* (scaffold 12130) and raw reads from the *P. alecto* genome (Fig. S1). No IFN genes were identified in the region 89 kb upstream of *IFN-β* (proximal 5′ end) or 94 kb downstream of *IFN-ε* (most 3′ end).

The *P. alecto* type I IFN locus was compared with the corresponding region from the genomes of 10 other vertebrates. The size of the IFN locus ranged from ∼25 kb in fish to 1 Mb in pig, with a trend toward increasing size through evolution as shown in Fig. 1. The only two exceptions were chicken and bat, both of which have shorter IFN loci of 30 kb and 250 kb, respectively.

A total of 10 genes with intact ORFs, including three *IFN-α* genes, one *IFN-β*, one *IFN-ε* gene, and five *IFN-ω* genes, were identified in the assembled bat IFN scaffold. In addition, a single copy of *IFN-κ* was found on a separate scaffold in the *P. alecto* genome (scaffold 14). The pattern of a single copy of *IFN-κ* and *IFN-ε* is conserved across all species (8). Consistent with the expansion in the genomic size of the IFN locus, gene duplication has occurred in the vertebrate type I IFN family in a step-wise manner, from only four type I IFNs at the basal branch such as in fish to 42 in pig. However, bats do not follow this trend and have only 10 type I IFN loci, three of which are *IFN-α* genes. Of the species that contain *IFN-α* genes, bats have the fewest *IFN-α* family members compared with any other mammalian genome studied (Fig. 1).

The deduced amino acid sequences of the three bat *IFN-α* genes share a number of features with *IFN-α* genes from humans

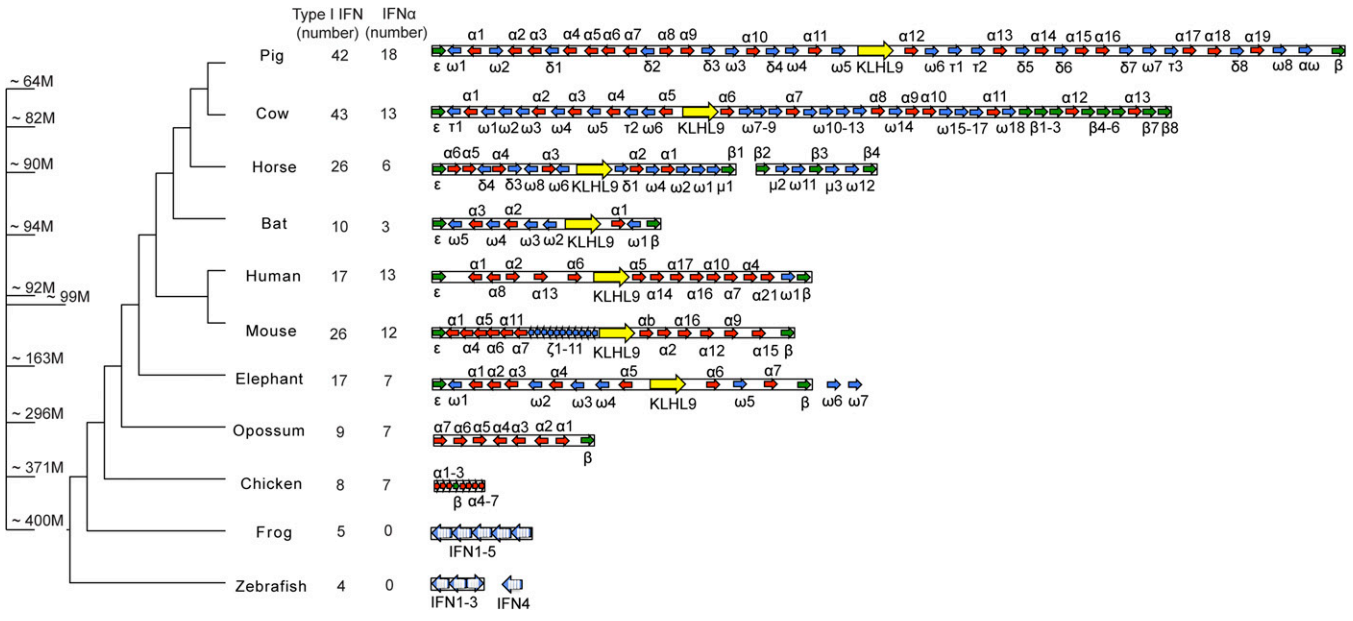

Fig. 1. Vertebrate type I IFN gene family among species. Type I IFN loci in selected vertebrate species (loci drawn to scale). IFN genes are annotated and labeled (not drawn to scale). The blocked arrows represent IFN ORFs, and directions indicate strand of the genes. *IFN-α* (red), *IFN-ε*, and *IFN-β* (green), other intron-less type I IFNs (blue), and the non-IFN gene, *KLH9* (yellow), are shown. The intron-containing fish and frog IFNs are shown in large blue blocked arrows, with white columns to indicate the exon/intron boundaries. The unplaced IFN containing fragments outside the major IFN locus for some species are also shown. The numbers of type I IFNs (including *IFN-κ*) and *IFN-α* counts for each species are shown on the right. The phylogenetic tree on the left was drawn according to TimeTree, and the approximate divergence times are labeled (M, million years) (38).

and other mammals, including predicted signal peptides and conserved binding domains for IFN-αR1 and IFN-αR2 for activation of downstream signaling (Fig. S2*A*). They share 93–96% similarity to each other and 79–85% similarity to human *IFN-α* genes at the amino acid level.

The bat IFN locus contains an additional eight *IFN-α* loci that appear to be pseudogenes (*IFN-αP*). This number is larger compared with humans or mice, which each have five *IFN-αP*s. Nucleotide alignment of the bat *IFN-αP* sequences with the three presumably functional *IFN-α* genes show that many of the *IFN-αP*s contain conserved partial IFN-αR binding domains, consistent with the likelihood that they once encoded functional IFN-α proteins (Fig. S2*B*).

Evolution of Bat IFN-α Families. *IFN-α* and *IFN-ω* shared a common ancestor ∼130 Mya and are interspersed with each other on the mammalian IFN locus (28) (Fig. 1). To determine the evolutionary pressures responsible for the diversification of type I IFN genes, we performed an evolutionary analysis of *IFN-α* and *IFN-ω* families across eight mammalian species. The ratio of nonsynonymous (dN) to synonymous (dS) changes (dN/dS ratio) was measured to examine the selection pressures on the bat *IFN-α* and *IFN-ω* genes. For the bat ancestor, the dN/dS ratio was 0.54, which is similar to the selection pressures on ancestral *IFN-α* genes from other species including pigs (0.58), horses (0.34), humans (0.24), and mice (0.85). The purifying selection of bat *IFN-α* genes indicates its functional conservation and importance. Interestingly, positive selection for *IFN-ω* was observed at the ancestor of bats (dN/dS ratio = 1.07), and its selection pressure was higher than any nonbat mammalian type I IFN genes.

***IFN-α* Maintains a Constitutive and Ubiquitous Expression Pattern in Bat Tissues and Cells.** We then examined the *IFN-α* mRNA expression in comparison with *IFN-β* in tissues from three apparently healthy wild-caught *P. alecto* bats. As shown in Fig. 2*A*, *P. alecto IFN-β* was undetectable across all tissues tested with the exception of testes. In contrast, primers capable of detecting all three bat *IFN-α* genes demonstrated significant expression of *IFN-α* in all bat organs tested, with lung and brain the highest and wing the lowest (Fig. 2*A*). To determine whether the constitutive expression of *IFN-α* is *P. alecto*-specific, a second bat species, the lesser short nosed fruit bat (*Cynopterus brachyotis*) was also tested. Similar to the *P. alecto* tissues, *IFN-α* was expressed constitutively in tissues from *C. brachyotis* in contrast to undetectable levels of *IFN-β* across all tissues tested (Fig. S3*A*).

The inducibility of bat *IFN-α* and *IFN-β* was then compared in primary cell lines derived from nine different *P. alecto* tissues before and after transfection with polyI:C for 3 h. The responses of primary cells confirmed our finding from the bat tissues demonstrating that *IFN-α* maintains a constitutive expression pattern in unstimulated bat primary cells. However, upon polyI:C treatment, *IFN-α* was not significantly induced. This is in clear contrast to *IFN-β*, which was highly induced in polyI:C-treated bat cells (Fig. S3*B*).

To examine the production patterns of bat *IFN-α* and *IFN-β* in response to viral challenge, we used two bat viruses and a mouse paramyxovirus to infect *P. alecto* kidney cell line PaKiT03 cells. Both Hendra virus (HeV) and Pulau virus (PulV) are bat-borne viruses carried by *Pteropus* bats. Sendai virus (SeV; Cantell strain) is a mouse paramyxovirus and is used in IFN research because of its ability to induce type I IFN through the production of defective interfering particles (29). Only SeV infection resulted in significant induction of *IFN-β* ($P < 0.05$). The absence of *IFN-β* induction is likely the result of antagonism of the IFN-β response by bat-borne viruses as reported previously for HeV (30). In contrast, *IFN-α* was significantly induced by SeV ($P < 0.05$) but to a lesser extent compared with the induction of *IFN-β*. Infection of bat cells with the two bat-borne viruses, HeV and PulV, caused no change in the constitutive *IFN-α* expression pattern (Fig. 2*B*). RNA sequencing (RNAseq) data available from HeV-infected human (HEK293T) and bat (PaKiT03) cells was used to confirm our findings (31). In bat cells, the constitutive *IFN-α* expression pattern was confirmed by using read depth counts of *IFN-α* transcripts in uninfected cells and showed little change following HeV infection. In contrast, few *IFN-α* transcripts were detected in infected or uninfected human cells (Fig. S3*C*). As a comparison, read mapping of RNAseq data from uninfected human and bat cells failed to detect *IFN-β* in either cell line. To confirm that the bat cells were not harboring an unrecognized infection, we used BLASTX to query the RNAseq data for the presence of sequences corresponding to known pathogens. Among the 64 million paired end reads in our dataset, no transcripts showed significant homology to known viruses or microbes. Even unknown viruses would be expected to show some sequence similarity to known virus families, as described previously for RNAseq data from bat tissues (32). This further supports our conclusion that the constitutive expression of *IFN-α* in bats is not associated with active viral infection.

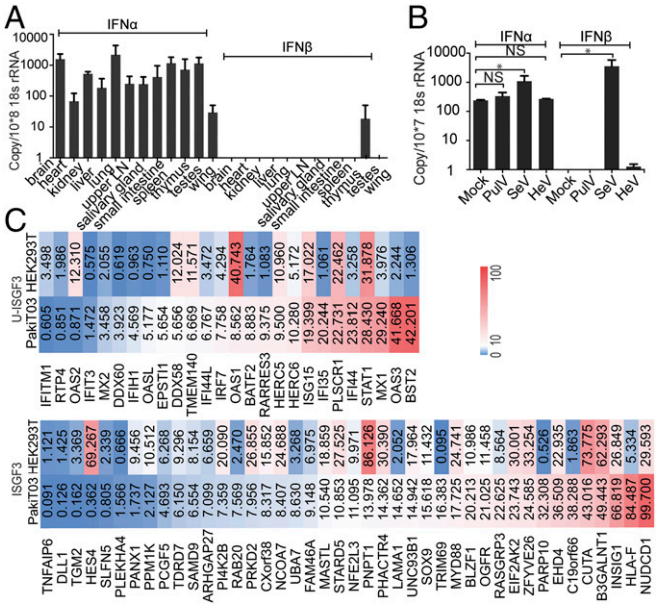

Fig. 2. Bat *IFN-α* has a constitutive and ubiquitous expression pattern. qRT-PCR detection of *IFN-α* and *IFN-β* mRNA expression in 12 *P. alecto* tissues ($n = 3$) (*A*). LN, lymph node. The error bars represent SD. (*B*) Bat PulV, HeV, and SeV were used to infect PaKiT03 cells. *IFN-α* and *IFN-β* mRNA expression was detected 6 h post infection. Two-sample *t* tests assuming unequal variance were used to compare IFN expression in response to viral infection. Data represent the mean and SE from three experiments (*$P < 0.05$). (*C*) Transcription profile of selected ISGs in uninfected PaKiT03 and HEK293T cells. Data illustrate average normalized fragments per kilobase of transcript per million mapped reads (FPKM) across four RNAseq replicates in PaKiT03 cells compared with HEK293T cells. ISGF3, ISG genes that are induced only by ISFG3; U-ISGF3, ISG genes that are induced by unphosphorylated ISGF3 (20).

Although the constitutive expression of bat IFN-α at the protein level has not been confirmed as a result of the absence of a bat-specific antibody, a high level of IFN-α protein expression would be expected to lead to the induction of ISGs. In human cells, continuous IFN-β exposure has been shown to lead to steady-state induction of the U-ISGF3–dependent proteins, with no sustained increase in other IFN-β–induced proteins (20). To determine whether the constitutive expression of IFN-α in bat cells resulted in induction of U-ISGF3–associated genes, we compared the expression of ISGF3-dependent and U-ISGF3–dependent transcripts in RNAseq data from uninfected human (HEK293T) and bat (PaKiT03) cells. Previous analyses describing U-ISGF3 and ISGF3-induced ISGs in human cells were used as the basis for distinguishing bat ISGs in the present study (20). Expression was

calculated using normalized read counts based on four replicates of RNAseq data from each cell line. Using a cutoff of 1.5-fold up-regulation between cell lines, 61.5% (16 of 26 genes) of U-ISGF3–dependent ISGs were expressed at a higher level in bat compared with human cell lines, compared with only 23.0% (6 of 26) that had higher expression in human cells. Conversely, 40.5% (17 of 42) of ISGF3-dependent ISGs displayed higher expression in human compared with bat cells, and only 33.3% (14 of 42) were higher expression in bat cells (Fig. 2*C*). The U-ISGF3–associated ISGs with the highest expression in bats included well-known antiviral proteins including bone marrow stromal cell antigen 2 (*BST2*; also known as tetherin) and *Mx1*. The expression of a subset of genes that were up-regulated in either bat or human cells was validated by using quantitative RT-PCR (qRT-PCR), confirming the pattern obtained from the RNAseq dataset (Fig. S4).

***IFNα2* and *IFNα3* Are the Main Constitutively Expressed Bat IFNs.** To test which bat *IFN-α* gene is constitutively expressed, TaqMan quantitative PCR (qPCR) assays were used to distinguish the three bat *IFN-α* genes in *P. alecto* tissues. *IFN-α* distribution among bat organs from three individual bats demonstrates that *IFN-α2* and *IFN-α3* are constitutively expressed in all organs tested, whereas *IFN-α1* is expressed to a lesser extent and only in a subset of tissues. *IFN-α2* and *IFN-α3* displayed a similar expression pattern across most organs with the exception of the thymus, in which *IFN-α3* was higher (Fig. 3*A*). These data confirm that *P. alecto* has three expressed *IFN-α* genes, of which *IFN-α2* and *IFN-α3* contribute to the majority of the constitutive expression of *IFN-α*.

For human *IFN-α1*, the unique promoter structure, and the simultaneous recruitment of IRF3 with the transcriptional coactivators CBP and p300, leads to a weak expression of endogenous *IFN-α1* (18). To explore whether constitutively expressed bat *IFN-α* genes also have unique promoters, we analyzed the proximal promoters of bat *IFN-α* genes. A region 200 bp upstream of the putative translation start which contains the three IRF binding modules in human and mouse *IFN-α* genes was chosen for this analysis (19, 33). The three modules were identified in bat *IFN-α1* and *IFN-α3*, and modules I and III were conserved with those of functional human *IFN-α* genes whereas module II was identical to that of human *IFN-α2*, which is nonfunctional (19). In contrast, the bat *IFN-α2* promoter contains mutations within module I and nucleotide insertions within modules II and III, which would render it unable to bind to IRFs (Fig. 3*B*). Promoter assays demonstrated that only *IFN-α1* and *IFN-α3* responded to IRF3 and IRF7, whereas *IFN-α2* failed to respond even in the presence of mitochondrial antiviral-signaling protein (MAVS), which is known to stimulate IRF activation (19) (Fig. S5). These findings are consistent with bat *IFN-α2* being regulated by factors other than IRF3 and IRF7 to maintain its constitutive expression pattern.

***P. alecto* IFN-α Proteins Are Functional.** To assess the functionality of *P. alecto* IFN-α proteins, plasmids encoding the three individual IFN-α ORFs were transiently transfected into human HEK293T cells. We chose HEK293T cells because of their high transfection efficiency, and also because the human IFN-αR cannot respond to bat IFN and trigger downstream signaling (Fig. S6). Cell supernatant was collected as IFN-α conditioned medium after confirming the successful expression of each protein (Fig. S7). Bat *IRF7*, *Mx1*, and *OAS1* were used as indicators of IFN-α functionality. Compared with untreated or vector mock-treated PaKiT03 cells, all three IFN-α proteins successfully induced ISGs, demonstrating that all three bat IFN-α proteins are potentially functional. As a positive control, recombinant bat IFN-β also induced ISG production in PaKiT03 cells (Fig. 4*A*). Although approximately similar quantities of each IFN protein were used, IFN-β resulted in higher ISG induction compared with the three IFN-α proteins ($P < 0.05$). This result may reflect a higher binding capacity to the IFN-αR as reported for human IFN-β (34).

As *IFN-α3* was the most abundant *IFN-α* in bat tissues, it was chosen to examine the antiviral activity of bat IFN-α. The antiviral

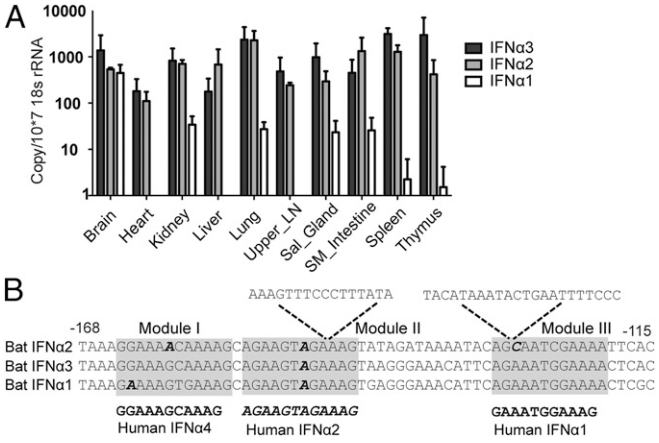

Fig. 3. *IFN-α2* and *IFN-α3* maintain a constitutive expression pattern. (*A*) *IFN-α* subtype mRNA expression in 10 *P. alecto* bat organs ($n = 3$) in sequence-specific TaqMan qPCR. The expression was normalized to the housekeeping gene 18S rRNA. The error bars represent SD. Sal_gland, salivary gland; SM_intestine, small intestine. (*B*) Sequence comparison of putative bat *IFN-α* gene promoters. The two sequence insertions in bat *IFN-α2* promoter are indicated. Three IRF binding modules that are important for human *IFN-α* induction were predicted in the bat promoter regions (shadowed) (18). The human reference modules are shown in bold and the reported nonfunctional module is in bold and italic (19).

activity of bat IFN-α3 was assessed on *Pteropine orthoreovirus* NB (PRV1NB)-infected PaKiT03 cells. PRV1NB is a biosafety level 2 reovirus carried by *Pteropus* bats that is easily cultured and tested for viral titer (35). IFN-α3 protected PaKiT03 cells from viral-induced cytopathogenic effect when applied 24 h before adding PRV1NB. IFN-α3 showed antiviral activity and *ISG56* inducibility in a dose-dependent manner, and the activity disappeared at 0.01 ng/mL (Fig. 4*B*). These results demonstrate that bat IFN-α3 could protect bat cells from PRV1NB viral infection.

Discussion

Type I IFNs provide the first line of defense against viral infection and are typically expressed only at low levels in unstimulated cells but are rapidly induced following infection. An increase in the size of the IFN locus has been accompanied by the evolution of a family of *IFN-α* genes that each display distinct roles in the antiviral immune response of most mammals. Paradoxically, bats, which are important reservoirs for a variety of viruses, have a contracted IFN locus and have only three functional *IFN-α* loci that are expressed constitutively in the absence of viral infection. The constitutive expression of bat IFN-α results in the up-regulation of a distinct subset of ISGs that may have implications for the ability of bats to coexist with viruses and resist DNA damage associated with flight.

The bat type I IFN locus was remarkably contracted in the bat genome at ∼250 kb compared with other eutherian mammals that range from 350 kb (mouse) to 1,000 kb (pig). The smaller genome size of flying mammals has been speculated to be related to the evolution of flight (36), with bats and birds having smaller genomes compared with other species (37). However, the contraction of the IFN locus is striking, with only three functional *IFN-α* genes in the bat genome compared with 7–18 *IFN-α* loci in other mammals. Contraction of the bat IFN locus appears to have occurred after the divergence of bats from ungulates ∼80 Mya (38). The presence of eight *IFN-α* pseudogenes provides further evidence for the contraction of the bat IFN locus from a large *IFN-α* family in the ancestral bat genome. The dN/dS ratio, indicative of purifying selection pressures shaping ancestral bat *IFN-α* emphasizes that the three functional bat *IFN-α* genes are conserved and functionally important to the host. In comparison, bat *IFN-ω* genes experienced positive selection at the

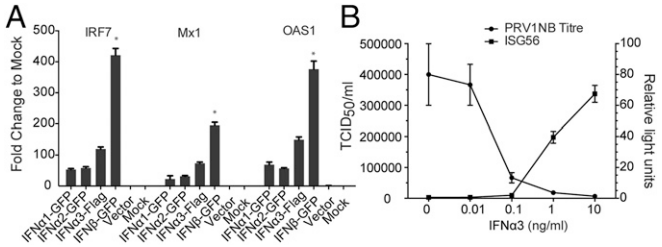

Fig. 4. Bat IFN-α proteins are functional. (*A*) Bat IFN-α proteins induce an ISG response. Bat IFN-α and IFN-β protein containing supernatant produced in HEK293T cells were used to treat PaKiT03 cells. Six hours later, cells were collected for qRT-PCR detection of mRNA expression of *IRF7*, *Mx1*, and *OAS1*. Supernatant from empty vector-transfected or mock-transfected HEK293T cells were used as controls. Data show fold changes compared with mock and represent the mean and SD from two experiments. The difference in ISG induction in response to IFN-β was calculated in comparison with ISG induction by each of the three individual IFN-α proteins (*$P < 0.05$). (*B*) Bat IFN-α3 blocked PRV1NB replication in a dose-dependent manner. IFN-α3 protein was added at varying doses to PaKiT03 cells before PRV1NB infection, and 50% tissue culture infective dose ($TCID_{50}$) assays were performed. In a parallel experiment, PaKiT03 cells were transfected with bat *ISG56* promoter reporter plasmid, and luciferase activity was analyzed after treatment with varying doses of IFN-α3. Fold activation was determined by dividing the relative light units of each experimental sample by the relative light units of media alone. Data represent the mean and SE of triplicate experiments.

ancestral branch, suggesting host–pathogen antagonism has continued through the long coevolutionary history of bats and viruses.

Type I IFN mRNA and proteins have been detected in tissues of healthy mice maintained in pathogen-free environments, but only in extremely low quantities (17). In humans, *IFN-α1* mRNA is detectible in healthy spleen, liver, and kidney, but not in other organs (13). Bats are unusual in that *IFN-α* mRNA is detectible across all organs from apparently healthy individuals of at least two bat species. In contrast, *IFN-β* is barely detectable. Furthermore, stimulation of bat cells with the dsRNA ligand polyI:C results in the up-regulation of *IFN-β* while the expression of *IFN-α* mRNA remained similar to that of unstimulated cells 3 h following stimulation. Our previous work also showed extremely low induction of *IFN-α* in response to polyI:C, up to a maximum of only approximately threefold at 9 h post transfection of bat lung cells (25). These findings suggest that *IFN-α* is not significantly up-regulated in response to cytoplasmic dsRNA sensing in bats. Nevertheless, the high baseline levels of *IFN-α* mean that substantial quantities of *IFN-α* mRNA can be detected even in the absence of immune stimulation. Similarly, *Rousettus aegyptiacus* lung cells had an apparent low level of constitutive *IFN-α* expression even before stimulation, and significant levels of *IFN-α* mRNA were not present until 8 h after polyI:C treatment (39). Thus, *IFN-α* in bats forms two layers of protection: the constitutive and the induced *IFN-α*. The human *IFN-α* response peaks at 2, 8, and 12 h following SeV infection (18), whereas, in bats, the *IFN-α* response is constitutively activated and further induced 8–9 h post infection (25). The two layers of the response are anticipated to provide bats with immediate protection but also allow them to react with a higher response when stimulated. Whether the two layers of the bat *IFN-α* response are dependent on each other or have different antiviral functions remains to be determined.

Very low levels of constitutively expressed IFN-α/IFN-β in humans and mice are believed to play a role in priming downstream responses, rather than having a direct role in antiviral immunity (16, 17). However, the ability of recombinant bat IFN-α3 to inhibit viral replication is consistent with constitutive IFN-α having a direct role in antiviral immunity in vivo. HeV has been demonstrated to block IFN production and signaling in bat cells (30), but does not affect the basal expression of *IFN-α*. RNAseq analysis supported our conclusion that bat *IFN-α* is constitutively expressed at a much higher level than human *IFN-α*, and is almost unaffected by HeV infection. Thus, the basal expression of bat *IFN-α* appears to be capable of avoiding the consequences of viral antagonism (at least by HeV and PulV) that leads to inhibition of the *IFN-β* response (30).

Constitutive expression of IFN-α would be expected to result in the corresponding induction of ISGs. To test this hypothesis, we used the available RNAseq dataset to compare expression of ISGs in human and bat cells (31). Human cells continually exposed to IFN-β express a distinct subset of ISGs that are driven by U-ISGF3, which leads to extended resistance to virus infection and DNA damage (20). The ISG response of bat cells appears to be enriched in ISGs associated with U-ISGF3. These ISGs were previously annotated, thus confirming they are counterparts to the corresponding human genes (21). Among the ISGs are well-characterized intracellular antiviral factors *BST-2* (tetherin), which has been reported to restrict replication of HIV, Ebola, and Marburg viruses, and *Mx1*, which is recognized as having broad-spectrum antiviral activity against many RNA viruses (including influenza virus) and some DNA viruses (40, 41). Such ISGs may provide a "switched-on" defense mechanism to blunt virus replication and potentially viral pathogenesis in bats. The ISGs driven by U-ISGF3 do not appear to mediate the acute inflammatory responses often associated with IFN responses and may therefore contribute to the ability of bats to tolerate high levels of IFN-α with no pathological consequences. Furthermore, this subset of ISGs has been linked to resistance to DNA damage in human cells (20). The evolution of a prolonged ISG response in bats may be yet another adaptation caused by the evolution of flight that has had inadvertent consequences for antiviral immunity (20).

Of the three functional *IFN-α* loci in the bat genome, all three show some level of expression in bat tissues and cells, but *IFN-α2* and *IFN-α3* account for the majority of the constitutive expression pattern. IRF3 and IRF7 drive IFN expression in humans and other species by binding to unique IRF modules in the promoter regions (18). Curiously, despite the high expression of *IFN-α2*, no intact IRF binding elements were present in the promoter region of this gene. Furthermore, only module I and III appear to be potentially functional in the promoter regions of *IFN-α3* and *IFN-α1*. Similar observations have been made in mouse *IFN-α13* and *IFN-ε*, in which the IFN promoters have lost modules for IRF binding but maintain a constitutive expression pattern in certain organs (thymus, spleen, and spinal cord for *IFN-α13*; female reproductive tract for *IFN-ε*) (42, 43). In both cases, IFN gene expression is independent of viral infection. It is possible that the constitutively expressed IRF7 of bats contributes to the constitutive expression of bat *IFN-α3* and *IFN-α1* (27). However, the absence of IRF binding modules in *IFN-α2* and the failure of IRF3 or IRF7 to induce *IFN-α2* is consistent with the possibility that other transcription factors drive expression of bat *IFN-α* genes. Differences in the induction of IFNs may provide the opportunity to avoid antagonism by viruses that target IFN production pathways (44).

IFN genes evolve by gene duplication and deletion, and the loss of genes indicates their functions have become redundant, as observed in birds, which have a highly contracted IFN family (45, 46). Natural selection can result in mutations that favor less than the complete repertoire of functional genes, often with favorable consequences. This has been termed the "less-is-more" hypothesis (47). The contraction of the type I IFN family in bats with corresponding changes in their expression patterns is consistent with this hypothesis. Bats use fewer *IFN-α* genes to efficiently perform the functions of as many as 13 *IFN-α*s in other species and have a system that is constitutively ready to respond to infection.

In summary, we present an evolutionarily unique bat type I IFN locus with the discovery of only three functional bat *IFN-α* genes. Although bats have fewer *IFN-α* family members, the constitutive and ubiquitous expression pattern of *IFN-α* in bats may provide bats with a highly effective system for controlling viral replication.

Methods

Bat Tissues and Cells. Tissues were collected from *P. alecto* and *C. brachyotis* bats as described in *SI Methods*. Details of the *P. alecto* primary and

immortalized cell lines and culture conditions are described in *SI Methods*. All animal experiments were conducted following guidelines approved by the Australian Animal Health Laboratory (AAHL) ethics committee (AEC1389 and AEC1557) or Singapore animal ethics committee [B01/12 (A4) 12].

Viral Infection. PaKiT03 cells were mock-infected or infected with HeV, Sendai virus, or PulV as described in *SI Methods*. IFN-α viral protection assays were performed in PaKiT03 cells as described in *SI Methods*.

IFN Locus Sequencing and Annotation. Detailed descriptions of the sequencing, annotation, and comparative analysis of the bat type I IFN locus with other species are provided in *SI Methods*.

Comparative and Evolutionary Analysis of the Mammalian Type I IFN Locus and IFN Genes. Comparative analysis of the bat IFN locus was performed with the corresponding genomic region from other vertebrates and is described in *SI Methods* and Tables S1–S9. Evolutionary analyses were performed by using sequence alignments of *IFN-ω* and *IFN-α* genes across a variety of vertebrates and is described in *SI Methods*.

Analysis of IFN and ISG Transcript Abundance. RNAseq datasets from *P. alecto* PaKiT03 and human HEK293T cells obtained from mock and HeV infection are described in *SI Methods* (31). Analyses to determine changes in transcript abundance of IFNs and ISGs are described in *SI Methods*. qRT-PCR validation of gene expression was performed on total RNA from tissues or cells as described previously (25) and described in *SI Methods*. Primers are listed in Table S10.

ISG Induction and Antiviral Activity of Bat IFN-α. Details of the cloning and expression of recombinant *P. alecto* IFN-α (IFN-α1–3) and IFN-β are described in *SI Methods*. The activity of the recombinant IFN-α proteins was determined by their ability to induce the production of *ISGs* and inhibit virus-mediated cytolysis as described in *SI Methods*.

Luciferase Promoter Assays. Details of the luciferase promoter assays used to test the ability of the three bat *IFN-α* genes to respond to IRF3 and IRF7 are described in *SI Methods*.

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